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Serum biomarkers as potential predictors of antitumor activity of cetuximab-containing therapy for locally advanced head and neck cancer

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Abstract

Objectives—We sought to identify biomarkers of antitumor activity in patients with locally advanced head and neck cancer treated with therapy containing cetuximab, an epidermal growth factor receptor (EGFR) inhibitor.

Materials and Methods—Patients with stage III-IVB head and neck cancer received cisplatin, docetaxel, and cetuximab (TPE) followed by radiotherapy, cisplatin, and cetuximab (XPE) and maintenance cetuximab in a phase II clinical trial. Serum and tissue biomarkers were examined for treatment-related changes and for association with clinical outcomes.

Results—Concentrations of 31 cytokines, chemokines and growth factors were measured before and after 3 cycles (9 weeks) of induction TPE using multi-analyte immunobead-based profiling (Luminex Corp., Austin, TX), with selected analytes validated by a single analyte enzyme-linked immunosorbent assay. Tumor biomarkers included phosphorylated signal transducer and activator of transcription-3 (pSTAT3), EGFR and human papillomavirus (HPV). Thirty-one patients had baseline biomarkers and 25 had paired samples, pre- and post-TPE. Adjusting for false discovery, 14 analytes including MCP1c, IP-10, Leptin, interleukin (IL)-5, Eotaxin, IL-6, G-CSF, CXCL5

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Conflict of interest statement

None declared

changed significantly post TPE induction. Serum vascular endothelial growth factor (VEGF) and IL-6 levels were associated with tumor response as assessed by positron emission tomography and progression-free survival, however, the association was not significant after adjustment for false discovery. Analytes were not associated with toxicities, smoking history, HPV status, EGFR amplification, or pSTAT3 tumor protein levels.

Conclusions—Baseline serum biomarkers, in particular VEGF and IL-6, were identified as potentially useful prognostic markers of cetuximab-containing therapy. Validation is warranted in future studies specifically designed to detect biomarker associations.

Keywords

head and neck cancer; cytokines; tumor markers; epidermal growth factor receptor; cetuximab; vascular endothelial growth factor; interleukin-6

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is an aggressive neoplasm that has been linked to alterations in immune and inflammatory responses, and angiogenesis (1–2). A better understanding of these aberrant responses might improve early detection and prognosis of SCCHN and provide novel therapeutic targets. We previously showed that alterations in serum biomarkers can be identified in patients with SCCHN. Although multiplexed panels of cytokines and chemokines had good diagnostic accuracy (85–90%) in our experience (2–4), these retrospective cohorts contained heterogeneously treated patients, limiting the prognostic and predictive information that could be gained.

Cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR), is active in SCCHN as single agent and in combination with chemotherapy or radiotherapy (5–6). However, despite the clinical benefits observed with EGFR-targeted therapies, there are no validated biomarkers of response to EGFR inhibitors. Recent data support a possible immune mechanism of action for cetuximab (7–11). Theoretically, targeting EGFR and downstream signaling molecules is likely to result in changes in local and systemic immune/inflammatory molecules that could be detected in the peripheral blood.

Our group has shown promising efficacy results in patients with head and neck cancer with a novel cetuximab-based regimen consisting of induction therapy with cisplatin, docetaxel, and cetuximab (“TPE”) followed by concomitant radiotherapy, cisplatin and cetuximab (“XPE”) and maintenance cetuximab (12). To explore serum biomarkers associated with clinical response to this novel, cetuximab-containing regimen, we performed serum multiplexed biomarker analysis before and after induction TPE using novel multi-analyte LabMAP profiling technology (Luminex™ Corp., Austin, TX). Luminex™ analysis allows for high-throughput measurement of a broad panel of analytes in a large set of specimens increasing clinical utility, with validation possible using standard ELISA. We hypothesized that an expanded panel comprised of multiple cytokines, chemokines, growth factors, and other tumor markers, which individually may show correlation with disease status, might provide higher diagnostic power if used in combination (2, 4). Furthermore, these biomarkers may provide insights into mechanisms of antitumor activity, or tumor progression, when correlated with clinical outcome.

Patients and Methods

Patient population

A phase II single arm clinical trial was carried out at the University of Pittsburgh (12). Eligibility criteria included locally advanced squamous cell or poorly differentiated carcinomas, unidimensionally measurable disease, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and adequate organ function, as previously described (12). The study protocol, including collection and analysis of biologic samples, was approved by University of Pittsburgh Institutional Review Board and all patients provided written informed consent. Patients were treated with a regimen consisting of docetaxel 75 mg/m² day 1, cisplatin 75 mg/m² day 1, and cetuximab 250 mg/m² days 1, 8, and 15 (after an initial dose of 400 mg/m² on cycle 1, day 1), termed “TPE”, and repeated every 21 days × 3 cycles, followed by radiotherapy to 70 Gy (2 Gy/day) with concurrent cisplatin 30 mg/m² and cetuximab 250 mg/m² weekly (“XPE”), and maintenance cetuximab for 6 months. Serum measurements were performed before and after the third cycle of induction (TPE) therapy. Tumor assessments with combined positron emission tomography/computed tomography (PET/CT) scanning were made after the third cycle of TPE and then approximately 8 weeks after XPE completion. Clinical trial results have been reported separately (12). Complete response (CR) by PET was defined as complete disappearance of [(18)F]fluorodeoxyglucose (FDG) uptake in all known tumor lesions, including primary tumor and neck nodes, as previously reported by our group (13). Of the 39 patients enrolled, blood samples were obtained from 31 patients at baseline and 25 patients post induction TPE therapy.

Collection and Storage of Serum

Ten milliliters of peripheral blood were drawn from subjects using standardized phlebotomy procedures and allowed to clot in red top vacutainers at room temperature. Sera were separated by centrifugation and all specimens were immediately aliquoted, frozen, and stored in a dedicated –80°C freezer. No freeze thawing was allowed prior to cytokine analysis.

Multiplex Serum Analysis

The LabMAP technology (Luminex™) combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology, allowing individual and multiplexed analysis of up to 100 different analytes in a single microtiter well. The LabMAP serum assays were done in 96-well microplate format according to the protocol by Biosource International (Camarillo, CA). A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 min with PBS/bovine serum albumin. The differentially dyed polystyrene microspheres served as a solid support for sandwich immunoassay.

The multiplexed analysis done by our group, along with the steps of development of LabMAP assays for cancer antigen (CA)-125, has been described previously in detail (2, 4, 13). In this study, we investigated a panel of a total of 31 cytokines, chemokines, growth factors, and other biomarkers (Table 2). Single analyte enzyme-linked immunosorbent assay (ELISA) validation for vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) was performed using Quantikine immunoassay kits (R&D Systems, Minneapolis, MN).

Determination of HPV, pSTAT3, and EGFR on baseline tumor tissue

Tissue microarrays (TMAs) were constructed from formalin fixed paraffin embedded tissue blocks from available pre-treatment, baseline tumor specimens. In situ hybridization (ISH) for human papillomavirus (HPV) DNA was performed with a proprietary pan selective probe set (Dako Cytomation, Carpinteria CA). Immunohistochemical (IHC) evaluation of

the remaining deparaffinized sections was performed using immunoperoxidase staining for p16 (p16INK4 mAb, BD Pharmingen, dilution 1:200) and scored semiquantitatively for each core. p16 immunoreactivity intensity of two or three in 70% or more cells was scored as positive (14).

For phosphorylated signal transducer and activator of transcription 3 (p-STAT3) determination by IHC, antigen retrieval was performed using Borg buffer (Biocare Medical) in the Biocare Decloaking chamber. Endogenous peroxidase was quenched with 3% Hydrogen peroxide (Fisher Scientific) for 5 minutes at room temperature. The slides were blocked with CAS block (Invitrogen) for 10 minutes, and incubated with anti p- stat3 (TYR705) rabbit monoclonal antibody (Cell Signaling) at 1:50 dilution for 75 minutes, followed by incubation with Mach 2 Rabbit Polymer H (Biocare Medical) for 30 minutes at room temperature. The slides were then washed with TBS buffer, incubated with Dako Substrate Chromagen (Dako) for 5 minutes at room temperature, and counterstained with Harris Hematoxylin. The staining was analyzed semiquantitatively based on the staining intensity (scale, 0 to 3) and the percentage of positive cells.

EGFR-fluorescence in situ hybridization (FISH) analysis was performed using standard method with the dual-color *EGFR* SpectrumOrange/CEP7 SpectrumGreen probe (Vysis, Downers Grove, IL) and paraffin pre-treatment reagent kit (Vysis, Inc., Downers Grove, IL), as previously described (15). Positive cases were considered those with high polysomy (4 gene copies in 40% of cells) or gene amplification (ratio EGFR gene/chromosome 7 > two or > 15 gene copies in > 10% of cells) (16).

Statistical analysis

Differences in characteristics between patients with and without available serum samples were examined for potential biases by recursive partitioning. Changes in serum analyte concentrations during TPE were tested with a signed rank test. Baseline serum biomarkers (available in a subset of patients) and the computed change in serum markers from baseline to after 3 cycles was compared between patients with and without complete response as assessed by PET using a two tailed Wilcoxon test. Recursive partitioning was also applied to the problem of classifying patients by PET response. Serum VEGF and IL-6 as measured by Luminex™ were examined for agreement with ELISA by estimating a regression model with a 95% prediction interval for the mean of mean of duplicate Luminex against the corresponding ELISA concentration. Progression-free survival (PFS) was estimated from the time of TPE initiation to the time of disease progression or last follow-up with Cox proportional hazards models. Overall survival (OS) was estimated from the time of TPE initiation to the time of death or last follow-up. Differential expression of analyte levels among patient groups defined by smoking history, HPV status, EGFR amplification, and pSTAT3 expression were tested with a two tailed Wilcoxon test. In general, results were interpreted as positive if the false discovery rate (FDR) was below 10% where the false discovery rate was estimated by the q value (17).

Results

Patient characteristics and clinical outcome

The characteristics of patients with a baseline serum biomarkers (n=31) are summarized in Table 1. The response rate, PFS and overall survival of patients with baseline serum biomarkers (n=31) were not different than that of the entire phase II trial cohort (n=39). For the 31 patients, with a median follow-up for patients without disease progression of 34 months (range, 28 – 44 months), the PFS rate at 3 years was 61% (95% CI, 34%– 80%) and the OS rate at 3 years was 78% (95% CI, 58%– 90%). PET/CT scan for response assessment

was performed in 28 of 39 patients; with induction TPE 6 patients achieved a CR by PET and 22 patients had residual FDG uptake on repeat scan.

Luminex™ Validation by single-analyte ELISA

To investigate the reliability of the screening multiplex (Luminex™) assays, individual ELISA assays were performed for VEGF and IL-6, using the same sera as for Luminex. Luminex™ results were highly correlated with single analyte ELISA for VEGF, and moderately correlated with IL-6 (Figure 1). In addition, VEGF and IL-6 levels were highly correlated with each other in individual patients' sera ($p < 0.001$, Spearman correlation coefficient 0.832).

Serum biomarkers changes and clinical outcome

A series of 31 analytes was measured using multiplex Luminex™-based ELISA assay from 100 μ l of serum (Table 2). For 25 patients from whom pre- and post-TPE induction serum samples were available, 14 analytes significantly changed with q values $< .10$ (Table 2). The top 5 ranked analytes were MCP1 ($q = 8.6E-5$), IP10 ($q = 8.6E-5$), Leptin ($q = 0.0001$), IL-5 ($q = 0.0002$), Eotaxin ($q = 0.0009$). None of the changes in the 31 analytes were associated with PET response.

Baseline serum analytes and clinical outcome

We used proportional hazards regression to test the association of the 31 analytes at baseline with PFS ($N = 29$). The following markers had raw p values of less than 0.10: IL-13, EGF, VEGF, and IL-6 (Table 3). However, when controlling for FDR at 10% none of the baseline values of the 31 analytes were associated with PFS (Table 3). Twenty-four patients had both post TPE response assessed by PET and baseline serum marker levels. Baseline VEGF, IL-6 and IP-10 concentrations among the 5 patients with a complete PET response were about half those of the 22 patients with residual metabolic activity and the unadjusted Wilcoxon p was .025, .024, and .033, respectively (Figure 2). When controlling for FDR at 10% none of the baseline serum analytes were associated with PET response. We applied recursive partitioning to classify PET response and VEGF was selected by the algorithm. Classification accuracy in this model was 91%. However, with leave-one-out cross-validation this model correctly classified 14 of 23 patients for a classification accuracy of 61% (95% CI = 41% – 81%).

Correlation of smoking status and other clinical parameters with serum analytes

Twelve patients had HPV positive tumors by HPV ISH and 13 patients had tumors positive by p16 IHC. There were no differences in serum analyte concentrations among HPV positive and negative patients (all $q > .10$). Similarly, there were no detectable baseline serum analyte differences by EGFR amplification by FISH (4 negative, 16 positive, all $q > .10$). Among the patients with baseline serum measurements there were 21 active smokers, 6 former smokers, and 4 never smokers. No differences in serum analytes were detected between these groups. Both OS and PFS were nearly identical for HPV-negative and HPV-positive patients, without correlation with serum or tissue biomarkers. As an exploratory analysis, tumor expression of p-STAT3 by IHC was investigated in relation to clinical outcome, and for association with serum analytes. These were individually tested for association with VEGF and IL-6 levels but no statistically significant associations were found (all q values $> .10$).

Discussion

EGFR inhibitors, such as cetuximab, can be successfully incorporated into combined modality regimens for the curative therapy of locally advanced SCCHN (1). However, despite promising results, the fraction of patients benefiting from the addition of cetuximab is small, and predictors of clinical activity besides the severity of rash after treatment have not been reproducibly identified. Thus, it is important to improve patient selection and identify the patients who would benefit the most from this targeted agent. Accumulating data suggest that cetuximab may mediate immune activation as a potential mechanism of clinical activity (7–11). Serum immune, angiogenic and growth factors are potentially appealing biomarkers which could be conveniently and rapidly obtained for prediction of cetuximab response.

We found low levels of VEGF and IL-6 levels measured at baseline to be consistent with better clinical outcomes. High levels of VEGF and IL-6 may be associated with poor metabolic response, as shown by residual FDG uptake on combined PET/CT, which we have previously found as an important predictor of poor PFS (13). Since these findings may be due to false discovery we suggest investigating these serum biomarkers in future trials for selection of candidates based on pretreatment levels, or in larger studies specifically designed to validate the findings we report here. We note that a recursive partitioning model for PET response by VEGF selected a cutoff with 91% classification accuracy. However, leave-one-out cross-validation, which is more likely to generalize to other patient populations than an unvalidated model, correctly predicted the PET response of only 14 of 23 patients. This gives an accuracy of 61% with a 95% confidence interval of 41% – 81%. Thus, the conclusions regarding prognostic potential of VEGF (and IL-6) may be explained by overfitting of our models to a small dataset. Because we conducted multiple testing, we recognize that these associations may be falsely positive. Indeed, the estimated expected false discovery rate for VEGF and IL-6 and IP-10 as predictors of PET response, as based on q value, was 28%. On the other hand, these cytokines are biologically relevant for SCCHN progression, and also the results are consistent with reports from another group (18). Byers et al reported that a panel of cytokines and angiogenic factors linked by hypoxia-associated responses, including VEGF, IL-4 and IL-8, osteopontin, growth-related oncogene-alpha, eotaxin, granulocyte-colony stimulating factor, and stromal cell-derived factor-1alpha, may correlate with outcome after induction therapy with carboplatin, paclitaxel and cetuximab, particularly in patients with HPV negative tumors (18). The induction regimen used in our study was similar but not identical since we used cisplatin (versus carboplatin) and another taxane (docetaxel versus paclitaxel). Also, patients in our study received cetuximab during subsequent radiotherapy with concurrent cisplatin and continued cetuximab for 6 months as maintenance therapy.

In the study reported here, it was interesting that strong correlation was observed between baseline VEGF and IL-6 ($p < 0.001$, Spearman correlation coefficient 0.817), since they are linked by the transcription factor STAT3, an oncogene and immunosuppressive marker in many cancers. IL-6 has been shown to activate STAT3, whereas STAT3 activates in turn VEGF and tumor angiogenesis (19). However, we found no association with tumor p-STAT3 and serum markers or clinical outcome, possibly suggesting that a potential source of the VEGF and IL-6 is from circulating immune/inflammatory cells not present in the tumor microenvironment.

Recent work supports a potential immune mechanism for cetuximab clinical activity (7–11), and this may be related to the serum levels of immunosuppressive cytokines, such as VEGF and IL-6 (19–21). Thus, the capacity to generate an immune response and to mediate cetuximab antitumor activity may be facilitated by low baseline levels of these cytokines.

Also, individual serologic growth, inflammatory, and angiogenesis markers have been correlated with disease status, including IL-1, IL-6, IL-8, GM-CSF, and VEGF; growth-regulated oncogene 1 (GRO-1); and HGF. Individually, IL-1 and IL-6 were found to promote survival and proliferation of SCC cells, and IL-8, GRO-1, VEGF, and HGF have been shown to contribute to angiogenesis, tumorigenesis, and metastasis. Furthermore, median serum concentrations of IL-6, IL-8, HGF, VEGF, and GRO-1 were found to be increased in patients with SCCHN and secreted by SCCHN cells (22–24). Our group has previously shown that multiplexed analysis of a cytokine biomarker panel could be used for the development of a screening test, in heterogeneously treated SCCHN or thyroid cancer patients with active disease or no evidence of disease after 3 years from curative therapy (2–4). Future studies in this area should also concentrate on repeated measurements during and after treatment, examining their associations with tumor response, relapse, complications, and survival. Because of the effect of smoking on various inflammatory mediators, we advocate more studies examining the role of biomarkers in association with smoking history which is a factor that appears to be associated with patient outcome (25).

The findings of this biomarker study have several limitations which need to be addressed in future clinical trials that will prospectively evaluate a small number of biomarkers to avoid false discovery. The collection of serum and tissue was not the primary purpose of this study, sample size was rather small, and selection of the panel of biomarkers was mostly driven by the purported cell signaling network and immune effects specific to cetuximab. However, since this was not a single-agent induction regimen any effect seen could have been due to the other drugs in the regimen (i.e. docetaxel and cisplatin).

Models to predict therapeutic response to EGFR inhibitors are of major interest and have been evaluated primarily in colorectal cancer (26–27). A study in patients with colorectal cancer treated with cetuximab reported longer overall survival in patients with VEGF reduction after therapy than in patients without (28). In a small number of patients with head and neck cancer treated with the combination of erlotinib and bevacizumab, higher ratios of tumour-cell phosphorylated VEGF receptor-2 (pVEGFR2) over total VEGFR2 and endothelial-cell pEGFR over total EGFR in pretreatment biopsies were associated with tumor response (29). Chung et al evaluated the potential value of a serum proteomic signature detected by mass spectroscopy (30) in predicting survival after treatment with cetuximab or an EGFR tyrosine kinase inhibitor in patients with non-small cell lung cancer, colorectal cancer and SCCHN (31). VEGF and IL-6 have been correlated with prognosis in several types of cancers, including SCCHN (19, 32–33). Whether VEGF and IL-6 are predictors of the efficacy of cetuximab-containing therapy or merely prognostic factors irrespective of treatment cannot be ascertained from our study. Our observations are hypothesis-generating and warrant validation in larger clinical studies, specifically designed to address this question. VEGF upregulation has been suggested as a mechanism of resistance to cetuximab (34). Thus, combined targeting of VEGF with cetuximab and bevacizumab may overcome tumor resistance to cetuximab alone. Such a study could include patients with low VEGF and/or IL-6, which might be expected to enhance the rate of CR to the induction, cetuximab based regimen. Alternatively, a trial design may incorporate anti-VEGF agents, such as bevacizumab. Given the poor prognosis of patients with high VEGF levels, it is possible that the addition of anti-VEGF therapy will enhance the antitumor efficacy of TPE. We are currently testing this hypothesis in a randomized, phase II clinical trial in locally advanced SCCHN at our institution.

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ELISA validation of VEGF and IL-6

FIGURE 1.

Luminex™ validation by single-analyte ELISA. To investigate the reliability of the screening multiplex (Luminex™) assays, individual ELISA assays were performed for VEGF and IL-6, using the same sera as for Luminex. Luminex™ results were highly correlated with single analyte ELISA for VEGF, and moderately correlated with IL-6. In addition, VEGF and IL-6 levels were highly correlated with each other in individual patients' sera ($p < 0.001$, Spearman correlation coefficient 0.832).

FIGURE 2.

Box and whisker plots of VEGF, IL-6 and IP10 by PET response.

The concentration levels of each analyte were compared for patients with and without a complete response (CR) by PET. P values for a two-tailed Wilcoxon test are shown above each plot.

Table 1

Characteristics of patients with baseline serum samples (n=31)

Median age, years (range)	54 (21–74)
Gender	
Male	27
Female	4
ECOG performance status	
0	22
1	9
Disease Site	
Hypopharynx	3
Larynx	4
Nasopharynx	3
Oral cavity	3
Oropharynx	16
Unknown primary	2
Stage	
III	2
IVa	26
IVb	3
T stage	
T0	2
T1	7
T2	9
T3	5
T4	8
N stage	
N0	1
N1	4
N2	23
N3	3
Smoking history	
Active smokers	21
Former smokers	3
Never smokers	4
20 pack-years or more	23
HPV status by ISH	
Positive	12
Negative	8
Unknown	11
EGFR status by FISH	
Positive	16

Negative	4
Unknown	11
Disease status	
Progression	10
Disease-free	19
Not evaluable	2

Table 2

Analytes examined by multiplexed analysis

Growth/ <u>Angiogenic Factors</u>	Interleukins	Chemokines/ <u>Cytokines</u>
VEGF	IL-1a	IFN- γ
FGF- β	IL-12p70	CD40L
*G-CSF	*IL-1Ra	TNF- α
HGF	IL-1b	MIP-1 α , MIP-1 β
*GM-CSF	IL-2, -4, -5, -6, -8	*MCP-1c
EGF	*IL-10, -13, -17	RANTES
		*EOTAXIN
		*CXCL5/EN
<u>Hormones</u>		
*Thrombopoietin		*IP-10
*Leptin		*I-TAC

* analytes that changed significantly after therapy ($q < 0.10$)

Table 3

Association of serum analytes at baseline with progression-free survival

Analyte	Coefficient*	p value	q value
IL13	-2.4436	0.01876	0.58156
EGF	1.7729	0.07082	0.72256
VEGF	1.7488	0.07337	0.72256
IL6	1.6436	0.09323	0.72256
CD40LT	1.4305	0.14435	0.75366
IL1Ra	1.4279	0.14587	0.75366
MIP1b	1.1762	0.23023	0.90150
IL5	-0.1893	0.25528	0.90150
IL17	1.1078	0.26518	0.90150
MIP1a	0.9767	0.32456	0.90150
IL1a1	0.8413	0.40450	0.90150
TNFaT	-0.8156	0.40895	0.90150
IP10	-0.6095	0.42191	0.90150
IL1b1	-0.7788	0.42743	0.90150
FGFbF	0.7852	0.43621	0.90150
Tpo	-0.6656	0.48948	0.92859
IFNgIF	0.6375	0.52290	0.92859
EOTAXIN	-0.6090	0.53918	0.92859
ITAC	-0.4859	0.61549	0.98702
MIP1c	-0.4205	0.67326	0.98702
IL10	-0.3871	0.69903	0.98702
IL12p70	-0.3337	0.73799	0.98702
GMCSF	-0.2953	0.76594	0.98702
GCSF	-0.2887	0.77222	0.98702
Leptin	0.2448	0.80654	0.98702
CXCL5EN	-0.1529	0.87839	0.98702
IL4	-0.1197	0.90468	0.98702
RANTES	-0.0842	0.93282	0.98702
IL2	-0.0754	0.93869	0.98702
HGF	0.0566	0.95518	0.98702
IL8CX	0.0109	0.99129	0.99129

* Standardized proportional hazards regression coefficient